© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb243062. doi:10.1242/jeb.243062

SHORT COMMUNICATION

Functional loss of ketogenesis in odontocete cetaceans

Michael J. Wolfgang[1,2,*], Joseph Choi[1] and Susanna Scafidi[3]

ABSTRACT

Odontocete cetaceans exhibit genomic mutations in key ketogenesis genes. In order to validate an inferred lack of ketogenesis made by observations from genome sequencing, we biochemically analyzed tissues from several odontocete cetacean species and demonstrate that they indeed do not exhibit appreciable hepatic β-hydroxybutyrate (βHB) or its carnitine ester. Furthermore, liver tissue exhibited significantly lower long chain acylcarnitines and increased odd chain acylcarnitines indicative of a decreased reliance on hepatic long chain fatty acid oxidation in these carnivorous mammals. Finally, we performed single molecule, real-time next generation sequencing of liver and brain RNA of *Tursiops truncatus* and demonstrate that the succinyl-CoA transferase required for acetoacetate catabolism is expressed in the nervous system. These data show that odontocete cetaceans have lost the ability to perform ketogenesis and suggest a hepatocentric coenzyme A recycling function rather than a predominantly systemic–bioenergetic role for ketogenesis in other ketogenic competent mammals such as humans.

KEY WORDS: Dolphin, Fasting, Ketogenic diet

INTRODUCTION

The consumption of a ketogenic, carbohydrate-limited diet requires adaptations to processes such as gluconeogenesis and ketogenesis to maintain sufficient fuel for the nervous system, especially for large brained mammals such as humans. The ketone bodies [acetone, acetoacetate and β-hydroxybutyrate (βHB)] are produced in the liver from acetyl-CoA derived primarily from fatty acid β-oxidation (Lee et al., 2016) and can change dramatically between the fed and fasted state (Cahill, 2006). Ketogenesis is thought to perform several important functions (Puchalska and Crawford, 2017). Ketone bodies can be used by most cells outside of the liver, including the brain, as an alternative oxidative substrate. This not only provides the brain with an alternative fuel but also staves off muscle wasting by reducing the need for amino acid carbon skeletons for gluconeogenesis. Additionally, the generation of ketone bodies in the liver is necessary for the liberation of coenzyme A from acetyl-CoA for the continued oxidation of fatty acids (Arima et al., 2021; Cotter et al., 2014). Without this, fatty acid oxidation would cease and would no longer enable gluconeogenesis (Fig. 1A). Finally, ketone bodies, particularly βHB, have been suggested to exhibit signaling properties well beyond their role in organismal metabolism (Newman and Verdin, 2017).

[1]Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. [2]Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. [3]Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

*Author for correspondence (mwolfga1@jhmi.edu)

M.J.W., 0000-0003-2349-8414

Received 22 June 2021; Accepted 29 September 2021

These putative important roles of ketone bodies have been challenged by the identification of multiple distant mammalian lineages that exhibit a genomic loss of hydroxymethyl-CoA synthase 2 (HMGCS2), an enzyme required for ketogenesis. This gene has been lost or made non-functional in the genomes of all odontocete cetaceans sequenced to date (dolphins and whales), elephants and Old World fruit bats (Jebb and Hiller, 2018). While it has been shown that fasting in fruit bats can be lethal, dolphins and elephants can fast for relatively long periods of time, suggesting an adaptive mechanism to maintain vital macronutrients. In fact, dolphins maintain a relatively high blood glucose concentration following a fast (Houser et al., 2021; Venn-Watson et al., 2011; Venn-Watson and Ridgway, 2007). How then can carnivorous dolphins consume a natural ketogenic diet yet maintain high circulating glucose concentrations during fasting in the absence of the generation of ketone bodies?

The loss of key ketogenesis genes in odontocete cetaceans suggests two mutually exclusive hypotheses. Either they do not perform ketogenesis or they have evolved an alternative ketogenic pathway utilizing, for example, Hmgcs1. In order to validate the inferred lack of ketogenesis made by observations from cetacean genome sequencing, we biochemically analyzed tissues from several odontocete cetacean species for hallmarks of ketogenesis. We found that in agreement with the genomic sequencing, odontocete cetaceans do not produce βHB and exhibit a liver acylcarnitine profile suggestive of a suppression in fatty acid catabolism and reliance on protein catabolism to fuel liver function. Finally, we utilized single molecule, real-time (SMRT) next generation sequencing of liver and brain RNA of *Tursiops truncatus* to gain insight into their unique metabolic adaptations. These comparative organismal data suggest a hepatocentric rather than systemic–bioenergetic role for ketogenesis in ketogenic competent mammals.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with the NIH's Guide for the Care and Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee. Mice (C57BL/6J) were housed in ventilated racks on a 14 h:10 h light:dark cycle and fed a standard chow diet (2018SX, Teklad Global) in a controlled environment with a room temperature of 21°C and 50% humidity. Livers were harvested from 9 week old male mice that were food deprived for 4 h (11:00 h to 15:00 h). The mice were first anesthetized with isoflurane until the toe pinch reflex was absent, then decapitated and the livers collected and frozen in liquid nitrogen. Samples were also obtained from livers of *Tursiops truncatus* (Montagu 1821), *Delphinus delphis* Linnaeus 1758 and *Grampus griseus* (G. Cuvier 1812), collected following stranding from the Atlantic ocean. Tissue samples from odontocete cetaceans were provided by Craig Harms (North Carolina State University) with permission from the National Marine Fisheries Services Table S1.

Journal of Experimental Biology (2021) 224, jeb243062. doi:10.1242/jeb.243062

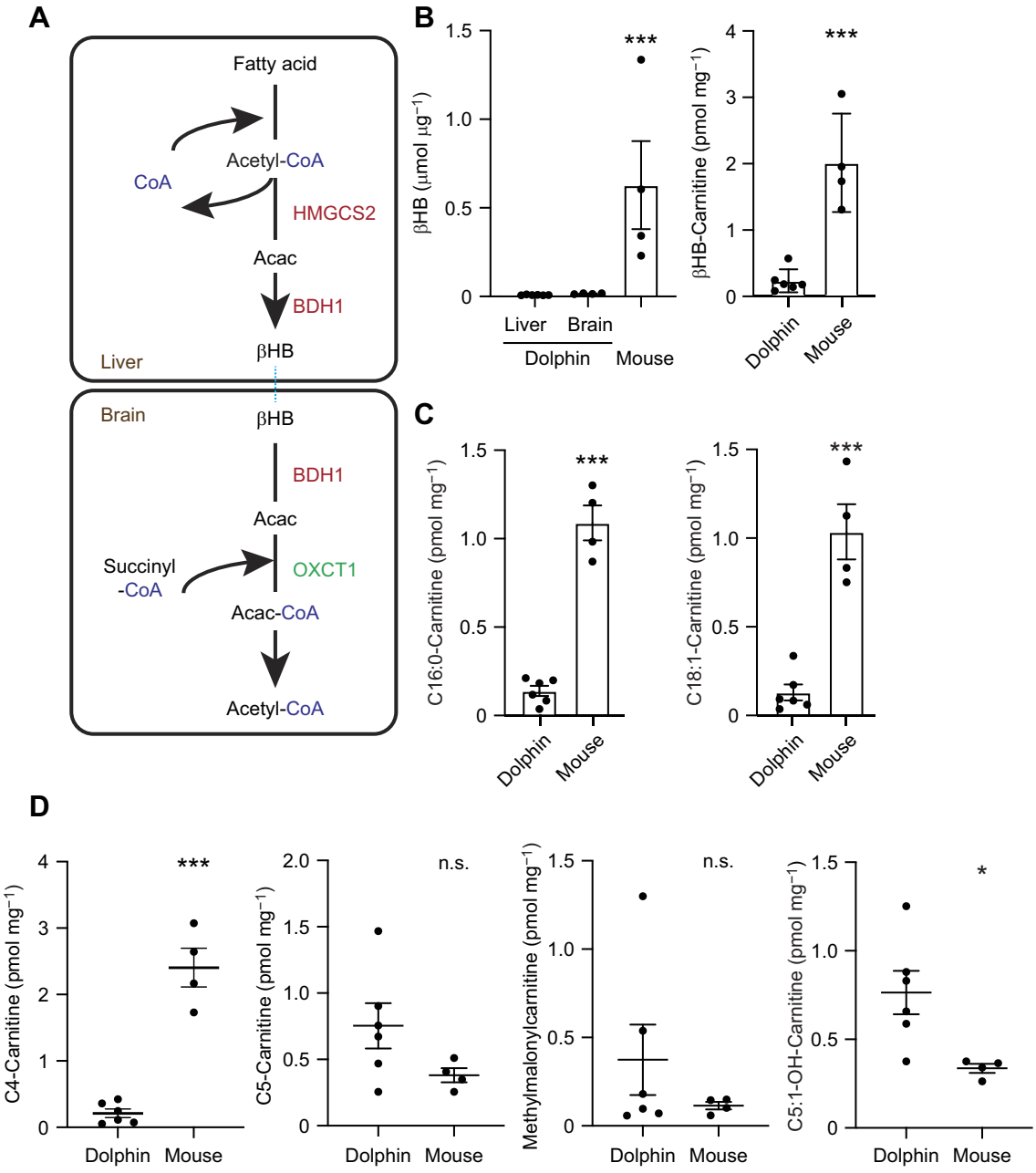

Fig. 1. *Tursiops truncatus* does not generate β-hydroxybutyrate (βHB) and suppresses hepatic fatty acid oxidation. (A) Schematic representation of ketone body metabolism. Red indicates that the corresponding gene is mutated in odontocete cetaceans; green indicates that the corresponding gene is present in odontocete cetaceans. CoA, coenzyme A; Acac, acetoacetate; HMGCS2, hydroxymethylglutaryl CoA synthase 2; BDH1, D-β-hydroxybutyrate dehydrogenase 1; OXCT1, succinyl-CoA:3-oxoacid-CoA transferase. (B) Liver βHB and βHB-carnitine, (C) long chain acylcarnitines and (D) additional acylcarnitines in *T. truncatus* brain/liver (n=6) and mouse liver (n=4). *P<0.05, ***P<0.01.

Metabolite measurements

Tissue βHB was measured by liquid chromatography–mass spectroscopy (LC-MS-MS) (Selen and Wolfgang, 2021). Liver samples were homogenized in an 80% methanol–water mixture, vortexed for 30 s and centrifuged at 13,000 ***g*** for 10 min at 4°C. The supernatant was then transferred to a new tube and placed into a speed-vac overnight until dried. The pellet was resuspended in 0.5 mol l^{-1} sodium hydroxide and used to quantify the protein concentration for data normalization by BCA assay (Thermo Scientific). Dried samples were reconstituted in 200 µl water just before LC-MS-MS analysis. A kinetex C18 column (2.6um, 50 mm, 2.1 mm, Phenomenex, Torrance, CA, USA) was used for liquid chromatography. Mobile phases were A: water+0.2% formic acid; and B: ACN+0.2% formic acid. The data were collected via a Shimadzu Nexera UHPLC (Shimadzu, Columbia, MD, USA), coupled to a 4500 triple quadruple mass spectrometer (A B Sciex, Redwood, CA, USA). The total run time was 5 min, with a flow rate of 0.2 ml min^{-1}. The gradient was applied as follows: 0% B at 0 min, 5% B at 0–4 min, 0 %B at 4–5 min with an injection volume of 2 µl. The retention time was observed at 1.64, with βHB at 102.9/58.8 (m/z). MultiQuant software (A B Sciex) was used to quantify the peaks against a 6-point-standard curve. Acylcarnitines were measured by standard methodology (Lee et al., 2016); 60 µl of 3 mol l^{-1} HCl in n-butanol was added to extracted samples,

incubated for 15 min at 65°C and then dried under liquid nitrogen. Butylated acylcarnitines were reconstituted in 100 µl of mobile phase acetonitrile/water/formic acid (H_2O/CH_3CN:/HCOOH, 80:19.9:0.1 v/v%). Samples were vortexed, transferred to a centrifuge filter, spun and transferred to an injection vial. Acylcarnitines were analyzed on an API 3200 (A B Sciex) operated in positive ion mode employing a precursor ion scan for *m*/*z* 85, which was generated as a characteristic product ion of a butyl ester of acylcarnitine species. Quantification of acylcarnitines was achieved by the Chemoview (A B Sciex) application. Metabolite concentrations were normalized to protein content (Table S2).

RNA sequencing

RNA was isolated from brain and liver of two *T. truncatus* samples using an RNeasy Mini Kit (Qiagen). SMRT next generation sequencing was used to generate and analyze full-length cDNA sequences. The processing and sequencing of RNA as well as bioinformatic analysis were performed by Novogene Corporation Inc. (Sacramento, CA, USA) (Table S3).

RESULTS AND DISCUSSION

In order to determine the presence or absence of ketogenesis in odontocete cetaceans, we obtained samples of liver from multiple species (*T. truncatus*, *D. delphis and G. griseus*) and measured the concentration of βHB by LC-MS-MS in comparison to that in mouse liver, serving as a positive control. We would expect the stress associated with stranding to further potentiate the release of ketogenic precursors even in animals consuming a ketogenic diet. While mouse liver contained abundant βHB, as expected, we could not find evidence of βHB from any odontocete cetacean liver (Fig. 1B; Table S2). The direct comparison of livers from stranded dolphins collected in the field and mice under well-controlled laboratory conditions certainly contains caveats. Regardless, we were unable to observe any βHB in any of the dolphin livers. Additionally, a recent study utilizing untargeted metabolomics did not observe βHB in the serum of fasted dolphins (Houser et al., 2021). These data show that, consistent with interpretations inferred from genomic sequencing, odontocete cetaceans are unable to generate βHB.

Dolphins consume a protein-rich ketogenic diet but do not produce ketone bodies. How then can they sustain high rates of hepatic fatty acid β-oxidation without recycling CoA via ketogenesis? To gain insight into this, we profiled liver acylcarnitines as a measure of protein and lipid intermediary metabolism. Consistent with a loss of βHB, odontocete cetacean livers did not contain appreciable βHB-carnitine esters, further demonstrating a lack of functional ketogenesis (Fig. 1B). Interestingly, odontocete cetacean livers contained surprisingly low concentrations of long even chain (C16 or greater) acyl-carnitine species such as palmitoylcarnitine and oleoylcarnitine in comparison to mouse liver, again with similar caveats to those described above (Fig. 1C). However, odontocete cetacean liver exhibited markers of protein catabolism such as isovalerylcarnitine and methylmalonylcarnitine (Fig. 1D). These data suggest that odontocete cetaceans do not rely heavily on hepatic fatty acid β-oxidation like other mammals and therefore have obviated their requirement for the hepatic CoA recycling facilitated by ketogenesis.

To gain a better understanding of the link between genomic structure and systemic physiology, we sequenced RNA from two brains and two livers from *T. truncatus* via long read SMRT technology. We were unable to recover mRNA sequence of an *Hmgcs2* gene in *T. truncatus*. However, *T. truncatus* has retained the enzymes responsible for ketolysis and we recovered the mRNA sequence of the *Oxct1* gene encoding succinyl-CoA:3-oxoacid-CoA transferase from *T. truncatus* brain. OXCT1 is required for ketone body utilization: βHB must first must be oxidized to acetoacetate; then, in order to oxidize acetoacetate, it must be esterified onto CoA (Fig. 1A). OXCT1 functions to transfer CoA from succinyl-CoA to acetoacetate to generate acetoacetyl-CoA. The mouse knockout of *Oxct1* results in perinatal lethality, but this lethality is mediated by ketoacidosis via an inability to clear acetoacetate and βHB rather than the loss of brain bioenergetics (Cotter et al., 2011, 2013). Excess ketogenesis brought about by the loss of insulin or *Oxct1* is acutely life threatening given the change in blood pH brought on by high concentrations of these weak acids. Why then would an animal evolve to eliminate genes of ketone body synthesis while retaining genes of ketone body oxidation? Ketogenesis is not the only metabolic pathway that generates acetoacetate. We would suggest the retention of *Oxct1* expression in the ketogenic incompetent *T. truncatus* is related to their requirement for acetoacetate catabolism from the breakdown of amino acids such as phenylalanine and tyrosine rather than ketone body catabolism per se.

The wealth of comparative genomic sequencing data has provided important insights into the evolution of unique adaptations across the animal kingdom (Huelsmann et al., 2019; Lopes-Marques et al., 2019). Dolphins have lost several important genes in lipid metabolism including *Acsm3*, *Dgat2l6*, *Fabp12*, *Pla2g2a* and *Thrsp* largely associated with unique adaptations in skin. The loss of key ketogenesis genes such as *Hmgcs2* and *Bdh1* in odontocete cetaceans suggests divergent hepatic function as well. Dolphins clearly perform gluconeogenesis as they can maintain or increase glucose levels following starvation (Houser et al., 2021; Venn-Watson et al., 2011; Venn-Watson and Ridgway, 2007). Dolphins may utilize gluconeogenic substrates that do not require the coupling of high rates of fatty acid oxidation to subsequent pyruvate carboxylation or they have evolved another mechanism to recycle CoA such as a mitochondrial thioesterase or mitochondrial uncoupling.

Do ketone bodies play an essential role as an alternative oxidative substrate in non-hepatic tissues during prolonged fasting? One might argue there is little experimental evidence supporting this notion. The clinical presentation of *Hmgcs2* mutations in humans and *Hmgcs2* knockout mice support a predominant role for ketogenesis in support of hepatic fatty acid oxidation rather than systemic bioenergetics (Arima et al., 2021; Thompson et al., 1997). Both humans and mice with *Hmgcs2* deficiency show effects that resemble inborn errors in long chain fatty acid oxidation, i.e. fasting-induced hypoketotic hypoglycemia, but not an obvious neurological energy crisis. Perhaps the predominant role for ketogenesis is to recycle coenzyme A in the liver to enable high rates of fatty acid oxidation in hepatocytes with a limited capacity for TCA cycle oxidation of acetyl-CoA. Ketogenesis and ketolysis must be coordinated during fasting in humans to prevent ketoacidosis, but further experimentation is required to elucidate the bioenergetic role/requirement of ketone bodies in ketogenic competent mammals.

Acknowledgements

The authors would like to thank Dr Craig Harms (North Carolina State University), Dr Vicky Thayer (North Carolina Division of Marine Fisheries) and Ainsley Smith (National Oceanic and Atmospheric Administration) for providing cetacean tissue samples. The authors would also like to thank Ebru Selen for metabolite measurements.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.J.W.; Methodology: S.S.; Formal analysis: S.S.; Investigation: J.C., S.S.; Writing - original draft: M.J.W.; Writing - review & editing: M.J.W., J.C., S.S.; Supervision: M.J.W.; Project administration: M.J.W.; Funding acquisition: M.J.W.

Funding
This work was supported in part by a National Institutes of Health grant R01DK120530 and R01DK116746 to M.J.W. Deposited in PMC for release after 12 months.

References

Arima, Y., Nakagawa, Y., Takeo, T., Ishida, T., Yamada, T., Hino, S., Nakao, M., Hanada, S., Umemoto, T., Suda, T. et al. (2021). Murine neonatal ketogenesis preserves mitochondrial energetics by preventing protein hyperacetylation. *Nat Metab* **3**, 196-210. doi:10.1038/s42255-021-00342-6

Cahill, G. F., Jr. (2006). Fuel metabolism in starvation. *Annu. Rev. Nutr.* **26**, 1-22. doi:10.1146/annurev.nutr.26.061505.111258

Cotter, D. G., d'Avignon, D. A., Wentz, A. E., Weber, M. L. and Crawford, P. A. (2011). Obligate role for ketone body oxidation in neonatal metabolic homeostasis. *J. Biol. Chem.* **286**, 6902-6910. doi:10.1074/jbc.M110.192369

Cotter, D. G., Ercal, B., Huang, X., Leid, J. M., d'Avignon, D. A., Graham, M. J., Dietzen, D. J., Brunt, E. M., Patti, G. J. and Crawford, P. A. (2014). Ketogenesis prevents diet-induced fatty liver injury and hyperglycemia. *J. Clin. Invest.* **124**, 5175-5190. doi:10.1172/JCI76388

Cotter, D. G., Schugar, R. C., Wentz, A. E., d'Avignon, D. A. and Crawford, P. A. (2013). Successful adaptation to ketosis by mice with tissue-specific deficiency of ketone body oxidation. *Am. J. Physiol. Endocrinol. Metab.* **304**, E363-E374. doi:10.1152/ajpendo.00547.2012

Houser, D. S., Derous, D., Douglas, A. and Lusseau, D. (2021). Metabolic response of dolphins to short-term fasting reveals physiological changes that differ from the traditional fasting model. *J. Exp. Biol.* **224**, jeb238915. doi:10.1242/jeb.238915

Huelsmann, M., Hecker, N., Springer, M. S., Gatesy, J., Sharma, V. and Hiller, M. (2019). Genes lost during the transition from land to water in cetaceans highlight genomic changes associated with aquatic adaptations. *Sci. Adv.* **5**, eaaw6671. doi:10.1126/sciadv.aaw6671

Jebb, D. and Hiller, M. (2018). Recurrent loss of HMGCS2 shows that ketogenesis is not essential for the evolution of large mammalian brains. *eLife* **7**, e38906. doi:10.7554/eLife.38906

Lee, J., Choi, J., Scafidi, S. and Wolfgang, M. J. (2016). Hepatic fatty acid oxidation restrains systemic catabolism during starvation. *Cell Rep.* **16**, 201-212. doi:10.1016/j.celrep.2016.05.062

Lopes-Marques, M., Machado, A. M., Alves, L. Q., Fonseca, M. M., Barbosa, S., Sinding, M.-H. S., Rasmussen, M. H., Iversen, M. R., Frost Bertelsen, M., Campos, P. F. et al. (2019). Complete inactivation of sebum-producing genes parallels the loss of sebaceous glands in Cetacea. *Mol. Biol. Evol.* **36**, 1270-1280. doi:10.1093/molbev/msz068

Newman, J. C. and Verdin, E. (2017). beta-hydroxybutyrate: a signaling metabolite. *Annu. Rev. Nutr.* **37**, 51-76. doi:10.1146/annurev-nutr-071816-064916

Puchalska, P. and Crawford, P. A. (2017). Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. *Cell Metab.* **25**, 262-284. doi:10.1016/j.cmet.2016.12.022

Selen, E. S. and Wolfgang, M. J. (2021). mTORC1 activation is not sufficient to suppress hepatic PPARα signaling or ketogenesis. *J. Biol. Chem.* **297**, 100884. doi:10.1016/j.jbc.2021.100884

Thompson, G. N., Hsu, B. Y. L., Pitt, J. J., Treacy, E. and Stanley, C. A. (1997). Fasting hypoketotic coma in a child with deficiency of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase. *N. Engl. J. Med.* **337**, 1203-1207. doi:10.1056/NEJM199710233371704

Venn-Watson, S. K. and Ridgway, S. H. (2007). Big brains and blood glucose: common ground for diabetes mellitus in humans and healthy dolphins. *Comp. Med.* **57**, 390-395.

Venn-Watson, S., Carlin, K. and Ridgway, S. (2011). Dolphins as animal models for type 2 diabetes: sustained, post-prandial hyperglycemia and hyperinsulinemia. *Gen. Comp. Endocrinol.* **170**, 193-199. doi:10.1016/j.ygcen.2010.10.005

Table S1.

Click here to download Table S1

Table S2.

Click here to download Table S2

Table S3.

Click here to download Table S3